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DOI:
10.1016/j.jaci.2018.04.021

Document Version
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Citation for published version (APA):
Fazekas-Singer, J., Singer, J., Ilieva, K. M., Matz, M., Herrmann, I., Spillner, E., Karagiannis, S. N., & Jensen-Jarolim, E. (2018). AllergoOncology: Generating a canine anti-cancer IgE against the epidermal growth factor receptor (EGFR): Generating a canine anticancer IgE against the epidermal growth factor receptor. *Journal of Allergy and Clinical Immunology*. https://doi.org/10.1016/j.jaci.2018.04.021

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Letter to the Editor

AllergoOncology: Generating a canine anticancer IgE against the epidermal growth factor receptor

To the Editor:

Cancer immunotherapy with antibodies has revolutionized clinical oncology, giving hope for patients with cancer. Among these, cetuximab, a mouse-human chimeric antibody, targeting the human epidermal growth factor receptor (EGFR),[1] inhibits growth signaling and mediates antibody-dependent cell-mediated cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP)[E5,2] of tumor cells. Harnessing T_H2 immune responses against cancer is a subject in the emerging field of "AllergoOncology," taking advantage of the high capacity of IgE to trigger immune reactions against cancer.[3] Unlike Fcγ-receptors, all Fcε-receptors are activating.[3] Because fewer than 1 in 10 anticancer drugs translate from murine preclinical studies to human clinical trials,[4] we focused on dogs (*Canis lupus familiaris)* with spontaneously occurring cancer as potential efficacy models. Mammary carcinomas and osteosarcomas in dogs and humans frequently overexpress EGFR.[4,5] Also, human and canine immune systems are highly similar,[3] rendering canine clinical trials a promising alternative to rodent experiments.

We previously developed a caninized cetuximab IgG, "can225-IgG,"[6] which here we engineered into cetuximab IgE, "can225-IgE-λ," retaining the exact epitope specificity, but with the canine λ- and ε-constant domains. Can225IgE-λ was cloned using a ligation-free cloning technique[E13] and expressed in Expi293F cells on the basis of our previously published method[7] (Fig 1, *A*; see this article's Methods section in the Online Repository at www.jacionline.org). The affinity-purified can225IgE-λ had an apparent molecular mass of 235 kDa (Fig 1, *B*) and was correctly assembled (Fig 1, *C* and *D*) with low amounts of free chains.

We confirmed antibody specificity to recombinant human EGFR by Western blot (Fig 1, *E*), to native EGFR on human A431 breast cancer cells (Fig 1, *F*), to P114 canine mammary carcinoma (Fig 1, *G*) and D17 canine osteosarcoma (Fig 1, *H*) cells, but no binding to EGFR-negative CHO K1 (control) cells (Fig 1, *I*).

Next, we assessed FcεRI expression on effector cell lines. Both human promonocytic U937 and canine monocytic/macrophage-like DH82 cells showed moderate to high FcεRI expression (Fig 1, *J* and *K*). Furthermore, canine MPT-1 mast cells showed relatively lower FcεRI expression (Fig 1, *L*) compared with RBL-SX38 rat basophilic leukemia cells transfected with human tetrameric FcεRI (Fig 1, *M*). Because no cross-reactive antibody for canine FcεRII (CD23) was available, we evaluated CD23 expression on canine effector cell lines by RT-PCR. We observed a more than 1000-fold higher relative CD23 expression by MPT-1, compared with DH82 cells (Fig 1, *N*). The can225IgE-λ antibody bound to Fcε receptors on U937 cells (Fig 2, *A*), DH82 cells (Fig 2, *B*), and MPT-1 (Fig 2, *C*) cells.

Having shown tumor target specificity and recognition of immune effector cells by can225IgE-λ, we next used a tumor cell killing assay[E4] to quantify immune-mediated tumor cell killing by can225IgE-λ via flow cytometry. Tumor cells killed by cytotoxicity (ADCC) and phagocytosis (ADCP) were counted separately (for gating strategy, see Fig E1, *A*, in this article's Online Repository at www.jacionline.org). We first used human A431 as cancer target cells, U937 as effector cells, and human and dog cetuximab IgG[E5] and IgE versions. Both cetuximab IgG and IgE showed high tumor cell killing potential, mostly via ADCC (Fig 2, *D*) mediated by effector cells.

Canine IgE shows a 33 times weaker binding strength to the human FcεRI compared with human IgE. Accordingly, can225-IgE-λ–triggered tumor killing with human effector cells was significantly lower than that triggered by human cetuximab-IgE (Fig 2, *E*). None of the antibodies had a significant effect on the ADCP of tumor cells (see Tables E1 and E3 in this article's Online Repository at www.jacionline.org).

We furthermore evaluated whether can225IgE-λ could activate canine effector cells (Fig 2, *F* and *G*; Table E1). In a time-dependent manner (Fig E1, *K*), can225IgE-λ triggered significantly higher levels of ADCC by canine DH82 macrophages compared with can225IgG (Fig 2, *F*). Conversely, and in agreement with previous studies,[E7,8] can225IgG was significantly superior to IgE with regard to triggering tumor cell ADCP (see Table E4 in this article's Online Repository at www.jacionline.org).

In addition, we investigated the effects of an approach using 50:50 can225IgG and can225IgE-λ at the same total antibody amount as samples treated with individual antibodies. Combinational treatment performed comparable tumor cell ADCC to that by can225IgE-λ alone, but significantly better ADCC than that by can225IgG alone. ADCP was lower than that triggered by can225IgG, but higher than in can225IgE-λ monotherapy (Fig 2, *F*).

Overall, can225IgE-λ mediated higher levels of total tumor cell killing compared with those mediated by can225IgG alone. Moreover, combining can225IgG and can225IgE-λ was significantly superior to each isotype alone (Fig 2, *G*; see Table E2 and Table E4 in this article's Online Repository at www.jacionline.org).

Similarly, we evaluated the cytotoxic killing potency of can225IgE-λ by MPT-1 canine mast cells against A431 and measured specific tumor cell killing (Fig 2, *H*). Although ADCP assessment by flow cytometry was not possible in this experimental setting due to cell clumping, we could indeed confirm mast cell–mediated ADCC by fluorescence microscopy (Fig 2, *I*). Such effector functions may be due to can225IgE-λ specifically binding to IgE receptors (FcεRI on RBL-SX38 cells, Fig 2, *J*) and canine CD23, expressed on transfected CHO K1 cells (Fig 2, *K*).

Therefore, we established an *in vitro* system for quantification and side-by-side comparison of anticancer ADCC/ADCP triggered by cetuximab IgG, IgE, and their canine counterparts. Unlike in humans where κ:λ light chains are expressed in a ratio of 2:1, the average κ:λ ratio in dogs is 1:9.[E11] Hence, we engineered can225IgE-λ. Because canine IgE is able to bind human FcεRI, we could demonstrate dose-dependent binding of can225IgE-λ

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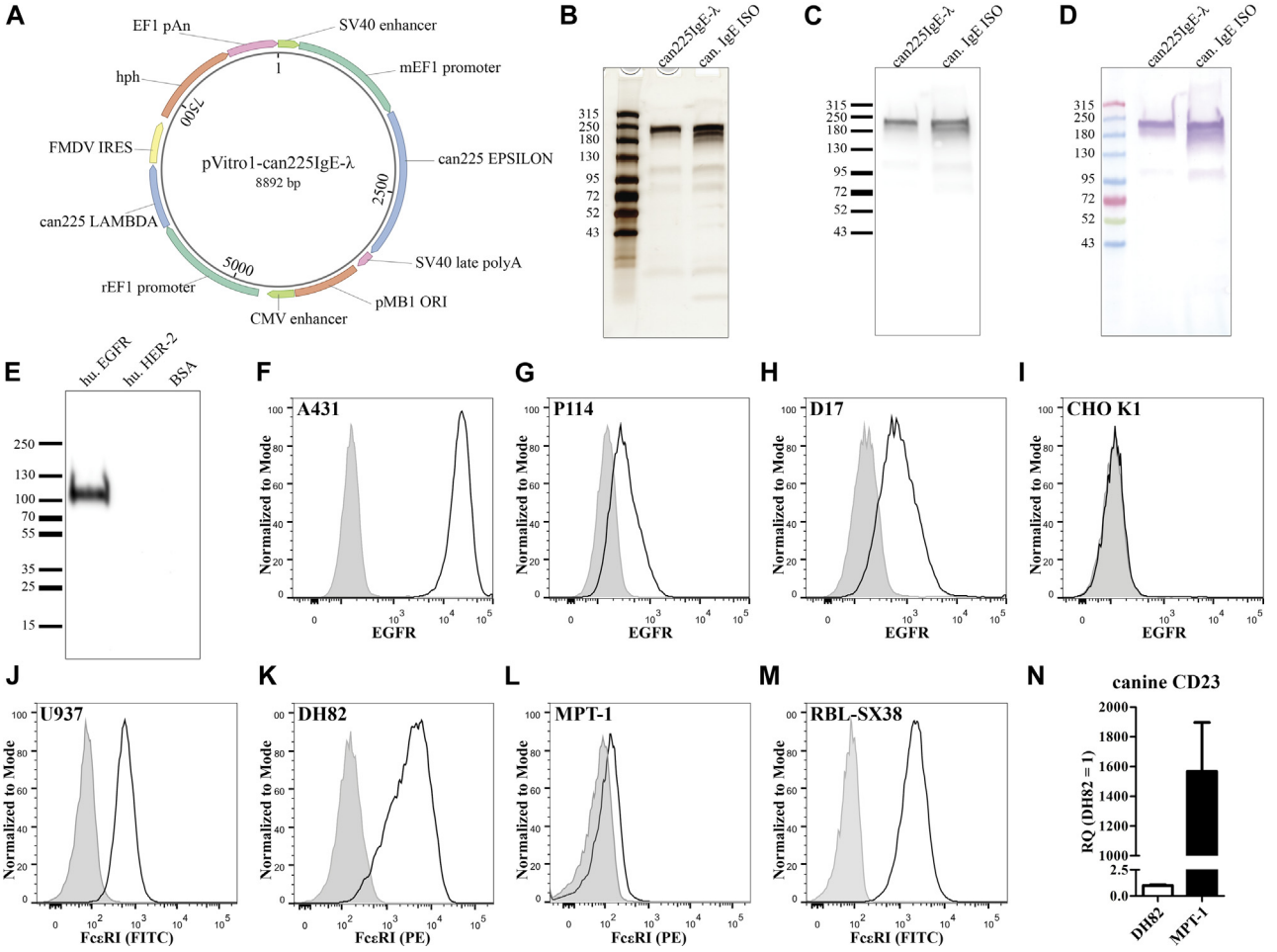

FIG 1. Expression and characterization of can225IgE-λ, and IgE-receptor expression on effector cell lines. Expression vector construct **(A)**, can225IgE-λ on a silver-stained protein gel **(B)**, and western blot developed with antidog IgE (Fc) **(C)** and with antidog light chain **(D)**. Binding of can225IgE-λ to human EGFR by Western blot **(E)** and to EGFR on human and canine target cell lines using EGFR-negative cell line CHO K1 for control **(F-I)**. FcεRI expression on human and canine effector cell lines **(J-M)** and relative CD23 expression of MPT-1 canine mast cells compared with DH82 monocytic/macrophage-like cells, normalized to beta-actin expression **(N)**. *CMV*, Cytomegalovirus; *HER-2*, epidermal growth factor receptor 2; *PE*, phycoerythrin; *SV40*, simian vacuolating virus 40.

on human U937 (Fig 2, *A*) in addition to the expected binding to canine FcεRI on DH82 and MPT-1 cells (Fig 2, *B* and *C*). Furthermore, we showed that DH82 macrophages express low levels of CD23 (Fig 1, *N*), but high levels of functional FcεRI (Fig 1, *K*), which is likely responsible for triggering IgE-mediated tumor cell killing (Fig 2, *F* and *G*), comparable to that mediated by human U937 monocytic effector cells. Using the canine DH82 macrophages, we were able to generate a species-relevant *in vitro* model system with which we confirmed the tumoricidal potencies of our canine antibodies, can225IgG or can225IgE-λ, alone, or combined.

It has been observed that human IgG triggers considerable levels of ADCP, whereas IgE mainly elicits ADCC by human monocytes/macrophages.[E7,8] We observed the same in the dog system and we also report significantly higher total tumor cell killing induced by IgE. We furthermore anticipated that IgE could act complementary to IgG, through using different Fc-receptors on effector cells. Coincubation with can225IgG and can225IgE-λ exerted higher tumor cell death, than did IgG or IgE treatment alone (Fig 2, *F*). Finally, we also demonstrate that can225IgE-λ is able to trigger specific ADCC against EGFR$^+$ tumor cells by canine MPT-1 mast cells (Fig 2, *H* and *I*).

Additional studies are needed to further evaluate these promising results with other IgG or IgE effector cells.[3,9]

This study presents the first *in vitro* functionally active canine anticancer IgE antibody for future clinical studies in dog cancer patients that may offer new options in cancer immunotherapy.[9]

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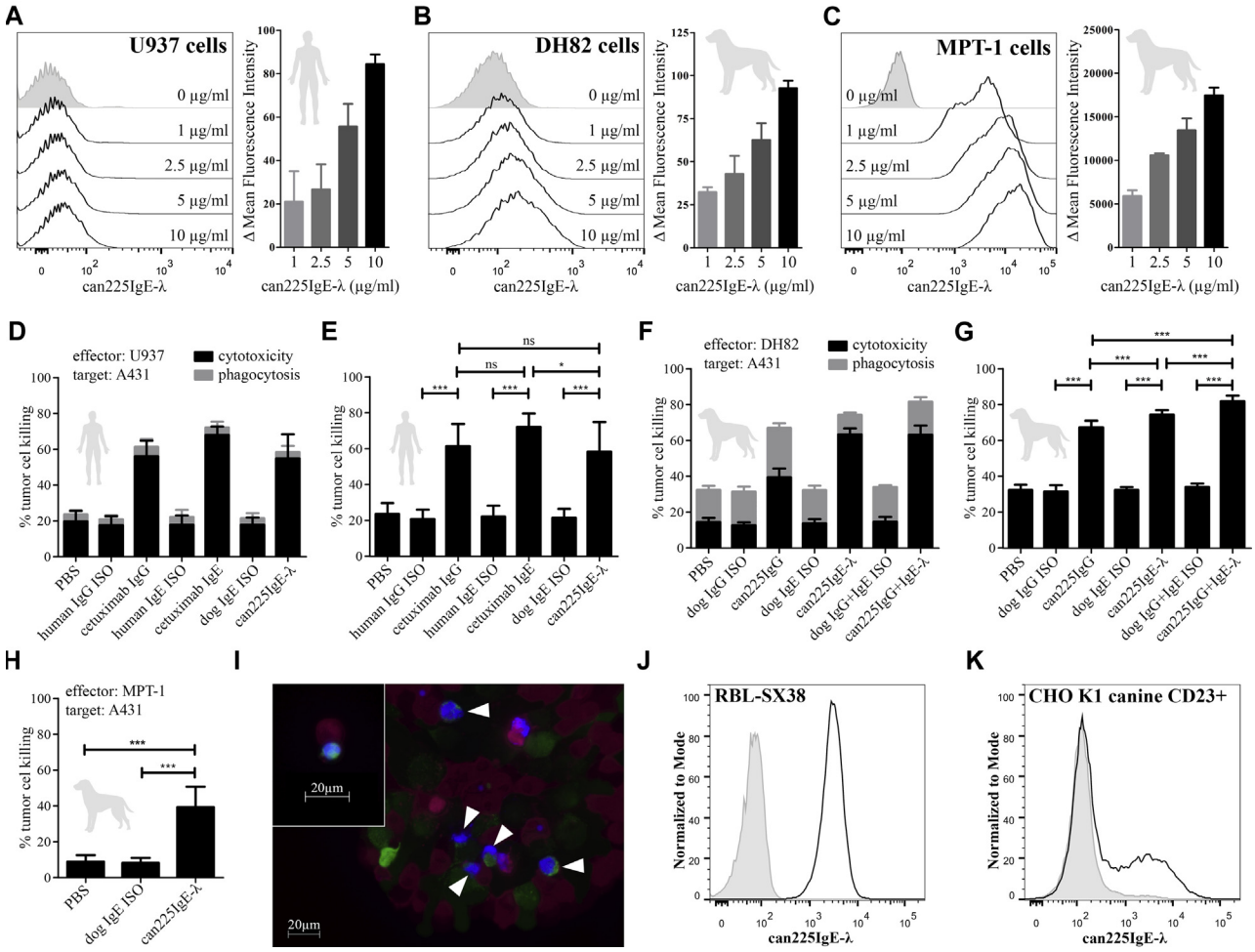

FIG 2. Functional characterization of can225IgE-λ. Dose-dependent binding of can225IgE-λ to Fcε-receptors on U937 human promonocytic cells **(A)**, DH82 canine monocytic/macrophage-like cells **(B)**, and MPT-1 canine mast cells **(C)**, with left panels depicting fluorescence histograms and right panels quantified delta mean fluorescence intensity values (n = 3). ADCC- and ADCP-triggering potency **(D)** and total tumor cell killing **(E)** induced by can225IgE-λ compared with other antibodies with human U937 cells and human A431 tumor cells as targets. ADCC- and ADCP-triggering potency **(F)** and total tumor cell killing **(G)** of can225IgE-λ compared head-to-head to its IgG counterpart can225IgG and to a combinatory can225IgG + IgE-λ treatment, using canine DH82 cells as effectors and A431 tumor cells as targets. ADCC-triggering potency of can225IgE-λ using canine MPT-1 mast cells as effector cells and human A431 as target cells **(H)**. Microscopic image of the ADCC assay depicted in panel *H*: can225IgE-λ–mediated cytotoxic killing of A431 cells (green) by MPT-1 mast cells (red) using DAPI (blue) as an indicator of dead cells (white arrowheads) **(I)**. Receptor-specific binding of can225IgE-λ to FcεRI expressed on RBL-SX38 cells **(J)** and to canine CD23 on transfected CHO K1 cells **(K)**. *DAPI*, 4′-6-Diamidino-2-phenylindole, dihydrochloride.

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This work was supported by the Austrian Science Fund (FWF) grants P23398-B11, W1205-B09, and SFB F4606-B28. The research was supported by the National Institute for Health Research (NIHR) Biomedical Research Centre based at Guy's and St Thomas' National Health Service Foundation Trust and King's College London (grant no. IS-BRC-1215-20006). The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR, or the Department of Health. The authors acknowledge support by Breast Cancer Now (147) working in partnership with Walk the Walk; the Medical Research Council (grant no. MR/L023091/1); Cancer Research UK (grant nos. C30122/A11527 and C30122/A15774); and CR UK/NIHR in England/DoH for Scotland, Wales and Northern Ireland Experimental Cancer Medicine Centre (grant no. C10355/A15587).

Disclosure of potential conflict of interest: J. Fazekas-Singer, J. Singer, and E. Jensen-Jarolim are inventors of can225IgG, which is claimed by the University of Veterinary Medicine Vienna, Austria. E. Jensen-Jarolim is founder and shareholder of Biomedical International R+D GmbH and inventor on nonrelated patents in allergen immunotherapy, antitumor vaccines, and biomedical imaging. S. N. Karagiannis is founder and shareholder of IGEM Therapeutics Ltd and coinventor of a patent on IgE antibodies for cancer therapy. J. Singer is coinventor of a nonrelated allergen immunotherapy patent. The rest of the authors declare that they have no relevant conflicts of interest.

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https://doi.org/10.1016/j.jaci.2018.04.021

RESULTS AND DISCUSSION

Affinity purification of can225IgE-λ

In contrast to previous reports,[E1] we did not succeed in purifying can225IgE-λ by Protein A affinity columns. This might be possibly due to 2 IgE isoforms in dogs, IgE1 having a high affinity toward *Staphylococcal* Protein A and IgE2 not at all binding to protein A.[E2]

ADCC and ADCP assay using EGFR- CHO K1 as target cells

The EGFR-negative CHO K1 cell line was used as control target cells. These cells were not recognized by can225IgE-λ (Fig 1, *I*). Consequently, CHO K1 cells were also not attacked by the human monocytic U937 cells (Fig E1, *C* and *D*) or the canine monocytic/macrophage-like DH82 cells (Fig E1, *E* and *F*).

Phenotypical characterization of DH82 and MPT-1 cells

DH82 cells appeared as single, round cells in culture and showed only 1 distinct population in flow cytometry scatter plots (Fig E1, *B*); 77.4% of DH82 cells were positive for CD16 (ΔMFI, 713), 99.9% for CD14 (ΔMFI, 12922), and 72.2% for CD80 (ΔMFI, 2797) (Fig E1, *G-I*).

DH82 cells transform over time from a monocyte- to a macrophage-like phenotype, associated with changes in CD14 and CD80 surface marker expression.[E3] However, both CD14 and CD80 expression levels, as well as morphology and the uniform population in flow-cytometric scatter plots, identified a monocytic phenotype,[E3] which was advantageous because the 3-color flow-cytometric assay had been established for monocytes.[E4]

METHODS

For proof-of-concept studies in the field of comparative immuno-oncology and AllergoOncology, the recombinant canine IgE antibody can225IgE-λ was developed against EGFR, which has 95% amino acid sequence homology and 92% identity among humans and dogs.[E5] The antibody was expressed in a transient expression system and characterized with respect to correct assembly, EGFR specificity, and binding to IgE receptors FcεRI and CD23. In addition, the functionality of can225IgE-λ was evaluated in a flow-cytometric ADCC and ADCP assay using human and canine monocyte-like cells, as well as canine mast cells as effectors against EGFR-overexpressing tumor and EGFR-negative control cells, as described below.

Cell lines, antibodies, and antigens

Cell lines. Human monocytic cell line U937 (CRL-1593.2), human epidermoid carcinoma cell line A431 (CRL-1555), canine osteosarcoma cell line D17 (CCL183), canine monocyte/macrophage-like cell line DH82 (CRL-10389), and Chinese hamster ovary cell line CHO K1 (CCL-61) were purchased from the American Type Culture Collection (Manassas, Va). Canine mammary carcinoma cell line P114 was a kind gift of G. Rutteman, University of Utrecht (Utrecht, The Netherlands). Canine mast cell line MPT-1[E6] was a kind gift of Prof. H. Matsuda, Tokyo University of Agriculture and Technology (Tokyo, Japan). U937 was cultured in RPMI 1640 GlutaMAX medium (Gibco, Thermo Fisher Scientific, Waltham, Mass) supplemented with 10% (v/v) FBS (Gibco, Thermo Fisher Scientific) and 100 U/mL penicillin and 100 μg/mL Streptomycin (Gibco, Thermo Fisher Scientific). A431 was grown in high glucose (4.5 g/L) Dulbecco modified Eagle medium (DMEM) GlutaMAX medium (Gibco, Thermo Fisher Scientific), supplemented with 10% FBS and 100 U/mL penicillin and 100 μg/mL streptomycin. D17 was grown in Minimum Essential Medium (MEM) GlutaMAX (Gibco, Thermo Fisher Scientific), augmented with 10% FBS and 100 U/mL penicillin and 100 μg/mL streptomycin. P114 was cultured in DMEM/F-12 GlutaMAX (Gibco, Thermo Fisher Scientific) media supplemented with 10% FBS and 100 U/mL penicillin and 100 μg/mL streptomycin. DH82 was cultured in low glucose (1 g/L) DMEM GlutaMAX medium (Gibco, Thermo Fisher Scientific), supplemented with 10% FBS and 100 U/mL penicillin and 100 μg/mL streptomycin. MPT-1 was grown in alpha-MEM GlutaMAX medium (Gibco, Thermo Fisher Scientific), supplemented with 10% FBS and 100 U/mL penicillin and 100 μg/mL streptomycin. CHO K1 was grown in Ham's F-12 Nutrient Mix GlutaMAX (Gibco, Thermo Fisher Scientific), augmented with 0% FBS and 100 U/mL penicillin and 100 μg/mL streptomycin. All cells were maintained in a humidified incubator at 37°C under a 5% CO_2 atmosphere.

Antibodies. Cetuximab (Erbitux) was purchased from Merck (Darmstadt, Germany). Rituximab (MabThera) was used as human IgG isotype control and was purchased from Roche (Basel, Switzerland). Cetuximab IgE[E7] was provided by Dr E. Spillner, Aarhus University (Aarhus, Denmark). MOv18-IgE[E8]—used as human IgE isotype control—was provided by Dr S.N. Karagiannis and Prof. H.J. Gould, King's College London (London, UK). Purified canine IgG (cat# IR-DG-GF, Innovative Research, Novi, Mich) was used as canine IgG isotype control and purified canine IgE (cat# P115, Bethyl Laboratories, Montgomery, Tex) as canine IgE isotype control. Can225IgG was produced as described by Singer et al.[E9]

Antigens. Recombinant human EGFR (extracellular domain) was purchased from ACROBiosystems (cat# EGR-H5222). Recombinant human epidermal growth factor receptor 2 (extracellular domain) was produced in-house by Lec-1 cells,[E10] which were a kind gift of D. Leahy from Johns Hopkins University School of Medicine (Baltimore, Md).

Cloning of can225IgE-λ

Cetuximab heavy-chain variable region sequence was fused with the canine immunoglobulin epsilon constant sequence (NCBI GenBank: AAA56797.1). Cetuximab light-chain variable region sequence was fused with the canine lambda (because of its higher prevalence in dogs than in humans).[E11] The light-chain constant sequence (NCBI Ref Seq: XP_013963655.1) can be found among the coding sequences in Table E5. The final sequences were synthesized by GeneArt in Strings DNA format (Thermo Fisher Scientific) and cloned into pCR-Blunt vector using Zero Blunt PCR Cloning Kit (Thermo Fisher Scientific). Coding sequences were recloned into pVitro1-hygro (Invivogen, San Diego, Calif) using a ligation-free polymerase incomplete primer extension cloning method adapted from Dodev et al.[E12] All primers used for polymerase incomplete primer extension cloning are listed in Table E6. The pVitro-hygro-can225-IgE-λ construct was then transformed into *Escherichia coli* Top10 (Thermo Fisher Scientific). Correct clones were verified using Sanger sequencing.

Production and purification of can225IgE-λ

Can225IgE-λ was produced according to Ilieva et al[E13] in human Expi293F cells using the pVitro1 vector (Fig 1, *A*) and purified with a custom affinity column using the $ÄKTA_{FPLC}$ system from GE Healthcare Life Sciences (Chicago, Ill). Custom anti-dog IgE column was made by crosslinking 1 mg goat anti-dog IgE (cat# NB7343, Novus Biologicals, Littleton, Colo) to HiTrap Protein G HP column (GE Healthcare Life Sciences) in the presence of 50-fold molar excess disuccinimidylsuberate (Sigma-Aldrich, St Louis, Mo). PBS, pH 7.4, was used as binding and washing buffer. Bound can225IgE-λ was then eluted with 0.1 M glycine (pH 2.5) followed by immediate pH neutralization using 1 M tris-HCl, pH 8.0.

PAGE and immunoblots

PAGE was run using tris-glycine–based 4% to 20% Mini-PROTEAN TGX gels (Bio-Rad Laboratories, Hercules, Calif), Laemmli loading buffer, and molecular weight markers Spectra Multicolor High Range Protein Ladder (Fig 1, *B-D*) and PageRuler Plus Prestained Protein Ladder (Fig 2, *A*) from

Thermo Fisher. The gels were loaded with 0.5 μg can225IgE-λ/canine IgE isotype or 1 μg recombinant human EGFR/recombinant human epidermal growth factor receptor 2/BSA and the proteins were subsequently detected either by silver staining or by immunoblot. Blocking and antibody dilutions were performed with 5% (w/v) skimmed milk powder dissolved in tris buffered saline, pH 7.2, + 0.1% (v/v) tween-20. Canine IgE was detected using horseradish peroxidase–labeled goat anti-dog IgE (cat# 7346, Novus Biologicals) at 1:5000 for 1 hour, or by 0.5 μg/mL can225IgE-λ for 2 hours at room temperature. Blots were developed with Clarity Western enhanced chemiluminescence substrate (Bio-Rad) using the VersaDoc Imaging System (Bio-Rad). Light chains were detected using 0.5 μg/mL anti-dog light-chain antibody (cat# A40-124A, Bethyl Laboratories) followed by alkaline phosphatase-labeled donkey anti-goat IgG detection antibody (cat# A16008, Thermo Fisher) at 1:2000, and subsequently visualized using 1-Step NBT/BCIP substrate solution (Thermo Fisher).

Determination of the molecular mass

The apparent molecular mass of can225IgE-λ on silver-stained PAGE was determined using GelAnalyzer 2010a software (Istvan Lazar and Dr Istvan Lazar). Spectra Multicolor High Range Protein Ladder (Thermo Fisher Scientific) was used as a reference.

Flow-cytometric binding assays

Specificity testing. A431, D17, P114, and CHO K1 cells were detached using Accutase solution (Sigma-Aldrich). A total of 3×10^5 cells/tube were stained with 10 μg/mL can225IgE-λ (or isotype control) for 30 minutes on ice followed by 10 μg/mL fluorescein isothiocyanate (FITC)-labeled goat anti-dog IgE (cat# NB7345, Novus Biologicals) for 30 minutes on ice in a light-protected environment. The cells were analyzed on a 3-laser FACSCanto II flow cytometer (BD Biosciences, Franklin Lakes, NJ).

FcεRI staining of effector cells. DH82 and MPT-1 (3×10^5 per test) were stained with 10 μg/mL phycoerythrin-labeled anti-mouse FcεRI (clone MAR-1) (cat# 12-5898-83, eBioscience, Santa Clara, Calif) or appropriate isotype controls. U937 and RBL-SX38 cells (3×10^5 per test) were stained with 10 μg/mL anti-human FcεRI (clone CRA-1) (cat# 14-5899-82, eBioscience, Santa Clara, Calif), or mouse IgG2b isotype control, detected by 10 μg/mL FITC-labeled goat anti-mouse IgG (cat# F0479, DAKO, Agilent Technologies, Santa Clara, Calif).

Fc-binding assay. U937, DH82, and MPT-1 cells (3×10^5 per test) were incubated with can225IgE-λ at different concentrations (1, 2.5, 5, and 10 μg/mL) for 30 minutes at 37°C. Bound can225IgE-λ was detected with 10 μg/mL FITC-labeled goat anti-dog IgE.

Receptor-specific binding of can225IgE-λ. RBL-SX38 or canine CD23-transfected CHO K1 cells (3×10^5 per test) were incubated with 10 μg/mL can225IgE-λ for 30 minutes. Following a washing step, bound can225IgE-λ was detected with 10 μg/mL FITC-labeled goat anti-dog IgE.

Characterization of DH82 cells. DH82 cells (passages 16-27, 3×10^5 per test) were stained with APC-labeled anti-human CD14 clone Tük4 (cat# MHCD1405, Thermo Fisher)/FITC-labeled anti-human CD16 (clone LNK16) (cat# MA1-19611, Thermo Fisher)/phycoerythrin-labeled anti-mouse CD80 (clone 16-10A1) (cat# 12-0801-82, eBioscience), or the respective controls. Cells were incubated with antibodies at concentrations according to the manufacturer's instructions for 30 minutes and washed before acquisition.

Real-time PCR analysis of canine CD23 expression

Total RNA of 5×10^6 DH82 and MPT-1 was isolated using the RNeasy Mini Kit (cat# 74104, Qiagen, Venlo, The Netherlands) and transcribed using the SuperScript II reverse transcriptase (cat# 18064022, Thermo Fisher) along with oligo$(dT)_{12\text{-}18}$ primers (cat# 18418012, Thermo Fisher) according to the manufacturer's instructions. Real-time PCR was performed using SYBR Green PCR Master Mix (cat# 4309155, Applied Biosystems, Thermo Fisher). Primers for canine CD23: forward: CCCAGAGCTTGAACGAGAGAA, reverse: TCCTCGCCGAAGTAGTAGCAC, as described by Amagai et al.[E6] Primers for beta-actin: forward: ATTGCCGACAGGATGCAGAA, reverse: GCTGATCCACATCTGCTGAA. RT-PCR analysis was performed using 3 biological replicates (and 3 technical replicates of each biological replicate).

3-Color flow-cytometric ADCC and ADCP assay

Flow-cytometric ADCC/ADCP assays were performed using the method described by Bracher et al.[E4] A431 tumor cells were prestained with 5 μM of the green fluorescent dye carboxyfluoresceinsuccinimidyl ester for 10 minutes, 18 hours before the experiment. The tumor cells (70000/test) were incubated with 5 μg/mL antibody solution in assay buffer (RPMI 1640 GlutaMAX +2% (v/v) FBS) at 37°C for 30 minutes. Next, 210,000 U937 cells/tube were coincubated with the tumor cells at 37°C for 2.5 hours. Following the incubation, U937 were labeled with APC-conjugated anti-human CD89 antibody (cat# 354106, BioLegend, San Diego, Calif). Dead cells were detected using the blue fluorescent DNA dye 4′,6-diamidino-2-phenylindole (cat# D1306, Thermo Fisher), few seconds before acquisition on the FACSCanto II.

When using DH82 or MPT-1 as effector cells, both tumor and effector cells were stained with cell-tracking dyes 18 hours in advance to the treatment antibodies and coincubation: Tumor cells were prestained with carboxyfluoresceinsuccinimidyl ester (as described earlier), whereas DH82 and MPT-1 were prestained with 1 μM CellTrace Far Red (Thermo Fisher) for 10 minutes. Because effector cells were already prelabeled in this case, no CD89 staining was needed to tag the cells after the coincubation of the tumor and effector cells. All assays were performed at least 3 times using 3 technical replicates per condition. Data were analyzed using FlowJo V10.0.7 (Flow Jo LLC, Ashland, Ore).

Fluorescence microscopy

Samples were prepared as described in the 3-color flow-cytometric ADCC and ADCP assay section using MPT-1 canine mast cells and A431 tumor cells. Fifteen microliter of the nonfixed cell suspension was mounted on a glass slide immediately after 4′,6-diamidino-2-phenylindole staining, and fluorescence microscopy pictures were recorded 20 minutes within mounting using an Axioplan 2 fluorescent microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) with a 20× air objective.

Generation of canine CD23-expressing CHO K1 cells

CHO K1 cells were transfected with pcDNA3.3-TOPO (Thermo Fisher) containing the coding sequence of canine CD23 (back-translated from the amino acid sequence disclosed under GenBank accession number AAN20273.1) using Lipofectamine 3000 (Thermo Fisher) according to the manufacturer's instructions. Forty-eight hours after transfection, 1 mg/mL G418 (cat# CP11.3, Carl Roth) was added and selection pressure was maintained over 3 weeks before proceeding to IgE-binding assays.

Statistical analyses

Statistical analyses were carried out using GraphPad Prism v5.00 for Windows (GraphPad Software, Inc, La Jolla, Calif). Fig 2, *A-C*, shows mean values + SEM, and Fig 2, *D-H*, and Fig E1, *C-F*, depict mean + SD. To calculate the statistical significance of induced killing between the different treatment groups (Table E3 and Table E4), we used 2-way ANOVA with Bonferroni posttest (phagocytosis and cytotoxicity). To compare the total killing induced by the antibodies (Fig 2, *E* and *G*, and Fig E1, *D* and *F*), we used 1-way ANOVA followed by Tukey multiple comparison test. Statistical significances are defined as $*P < .05$, $**P < .01$, and $***P < .001$.

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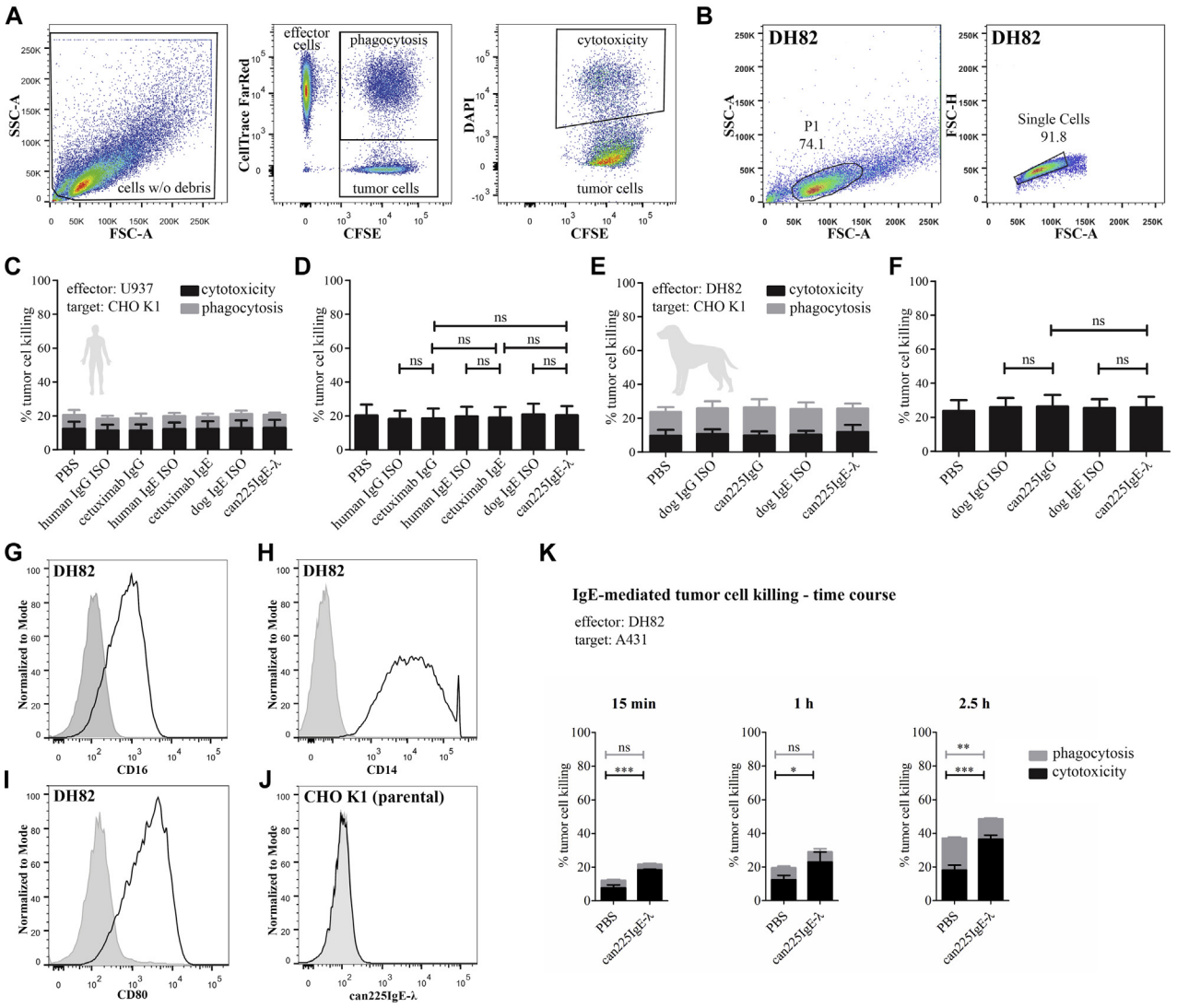

FIG E1. Gating strategies, ADCC and ADCP control assays, and phenotypical characterization of effector cells. Gating strategy in flow-cytometric ADCC and ADCP assays **(A)** and DH82 cell characterization **(B)**. ADCC- and ADCP-triggering potency **(C)** and total tumor cell killing **(D)** induced by can225IgE-λ compared with other antibodies in a negative control assay using U937 as effector and EGFR-negative CHO K1 as target cells. ADCC- and ADCP-triggering potency **(E)** and total tumor cell killing **(F)** induced by can225IgE-λ compared with other antibodies in a negative control assay using DH82 as effector and EGFR-negative CHO K1 as target cells. DH82 cell characterization in regard of CD16 **(G)**, CD14 **(H)**, and CD80 **(I)** expression, pointing toward a monocyte-like phenotype. Negative control experiment to Fig 2, *K*: nontransfected (parental) CHO K1 did not bind can225IgE-λ **(J)**. Evaluating IgE-mediated tumor cell killing over time confirmed previous literature reporting 2.5 hours as the optimal incubation time **(K)**. *CFSE*, Carboxyfluoresceinsuccinimidyl ester; *FSC-A*, forward scatter-area; *ns*, nonsignificant; *SSC-A*, side scatter-area.

TABLE E1. ADCC and ADCP triggered by different antibody treatments

U937 as effector cells							
Killing mechanism	**PBS**	**Human IgG ISO**	**Cetuximab IgG**	**Human IgE ISO**	**Cetuximab IgE**	**Dog IgE ISO**	**can225IgE-λ**
Cytotoxicity	20% ± 6.0%	18% ± 5.0%	56% ± 8.6%	18% ± 5.2%	68% ± 4.7%	18% ± 4.0%	55 % ± 14%
Phagocytosis	4.0% ± 2.3%	3.3% ± 2.4%	5.3% ± 4.3%	4.4% ± 4.0%	4.2% ± 3.3%	3.7% ± 2.9%	3.6% ± 3.4%

DH82 as effector cells							
Killing mechanism	**PBS**	**Dog IgG ISO**	**can225IgG**	**Dog IgE ISO**	**can225IgE-λ**	**Dog IgG + dog IgE ISO**	**can225IgG + can225IgE-λ**
Cytotoxicity	15% ± 2.3%	13% ± 1.7%	40% ± 4.9%	14% ± 2.4%	64% ± 3.4%	15% ± 2.8%	63% ± 5.1%
Phagocytosis	18% ± 2.3%	19% ± 2.9%	28% ± 2.6%	19% ± 2.5%	11% ± 1.3%	19% ± 1.1%	18% ± 2.5%

Depicted are mean values ± SDs in percent of the total number of tumor cells in the sample. n = 9 in all groups except combinational treatment with dog IgG and IgE isotype control and can225IgG+IgE, where n = 6.

ISO, Isotype control antibody; *PBS*, phosphate buffered saline, mock-treated control.

TABLE E2. Total tumor cell killing triggered by different antibody treatments

U937 as effector cells							
Treatment	**PBS**	**Human IgG ISO**	**Cetuximab IgG**	**Human IgE ISO**	**Cetuximab IgE**	**Dog IgE ISO**	**can225IgE-λ**
Total killing	24% ± 6.0%	21% ± 5.2%	61% ± 12%	22% ± 6.1%	72% ± 7.5%	21% ± 5.0%	58% ± 16%

DH82 as effector cells							
Treatment	**PBS**	**Dog IgG ISO**	**can225IgG**	**Dog IgE ISO**	**can225IgE**	**Dog IgG + dog IgE ISO**	**can225IgG + can225IgE-λ**
Total killing	32% ± 2.9%	32% ± 3.6%	67% ± 3.8%	32% ± 1.6%	74% ± 2.6%	34% ± 1.9%	82% ± 3.1%

Depicted are mean values ± SDs in percent of the total number of tumor cells in the sample. n = 9 in all groups except combinational treatment with dog IgG and IgE isotype control and can225IgG+IgE, where n = 6.

ISO, Isotype control antibody; *PBS*, phosphate buffered saline, mock-treated control.

TABLE E3. Detailed statistical analysis of antibody treatments in ADCC and ADCP assays using the human U937 as effector cells

	Cytotoxicity →						
Phagocytosis ↓	**PBS**	**Human IgG ISO**	**Cetuximab IgG**	**Human IgE ISO**	**Cetuximab IgE**	**Dog IgE ISO**	**can225IgE**
PBS		ns	***	ns	***	ns	***
Human IgG ISO	ns		***	ns	***	ns	***
Cetuximab IgG	ns	ns		***	***	*	ns
Human IgE ISO	ns	ns	ns		***	ns	***
Cetuximab IgE	ns	ns	ns	ns		***	***
Dog IgE ISO	ns	ns	ns	ns	ns		***
can225IgE	ns	ns	ns	ns	ns	ns	

Statistical analysis was calculated by 2-way ANOVA and Bonferroni posttest. Significance is determined as following: ns, nonsignificant ($P > .05$).
*$P < .05$.
***$P < .001$.

TABLE E4. Detailed statistical analysis of antibody treatments in ADCC and ADCP assays using the canine DH82 as effector cells

	Cytotoxicity →						
Phagocytosis ↓	**PBS**	**Dog IgG ISO**	**can225IgG**	**Dog IgE ISO**	**can225IgE**	**Dog IgG + dog IgE ISO**	**can225IgG + can225IgE**
PBS		ns	***	ns	***	ns	***
Dog IgG ISO	ns		***	ns	***	ns	***
can225IgG	***	***		***	***	***	***
Dog IgE ISO	ns	ns	***		*	ns	***
can225IgE	***	***	***	***		***	ns
Dog IgG + dog IgE ISO	ns	ns	***	ns	***		***
can225IgG +can225IgE	ns	ns	***	ns	***	ns	

Statistical analysis was calculated by 2-way ANOVA and Bonferroni posttest. Significance is determined as following: ns, nonsignificant ($P > .05$).
*$P < .05$.
***$P < .001$.

TABLE E5. Amino acid and coding DNA sequence of can225IgE-λ

Sequence description	Sequence
Can225IgE-λ EPSILON chain amino acid sequence *Legend:* **Bold**, dog immunoglobulin heavy-chain variable region leader (ACI25521.1) Underlined, 225 variable region Regular, dog immunoglobulin epsilon constant region	**MESVLGWVFLVAILQGVQG**QVQLKQSGPGLVQPSQSLSITCTVSGFSLTNYGVHWVRQSPGKGLEWLGVIWSGGN TDYNTPFTSRLSINKDNSKSQVFFKMNSLQSNDTAIYYCARALTYYDYEFAYWGQGTLVTVSAATSQDLSVFPLASCC KDNIASTSVTLGCLVTGYLPMSTTVTWDTGSLNKNVTTFPTTFHETYGLHSIVSQVTASGKWAKQRFTCSVAHAESTA INKTFSACALNFIPPTVKLFHSSCNPVGDTHTTIQLLCLISGYVPGDMEVIWLVDGQKATNIFPYTAPGTKEGNVTSTH SELNITQGEWVSQKTYTCQVTYQGFTFKDEARKCSESDPRGVTSYLSPPSPLDLYVHKAPKITCLVVDLATMEGMNL TWYRESKEPVNPGPLNKKDHFNGTITVTSTLPVNTNDWIEGETYYCRVTHPHLPKDIVRSIAKAPGKRAPPDVYLFLP PEEEQGTKDRVTLTCLIQNFFPADISVQWLRNDSPIQTDQYTTTGPHKVSGSRPAFFIFSRLEVSRVDWEQKNKFTCQV VHEALSGSRILQKWVSKTPGK
Can225IgE-λ EPSILON chain DNA sequence (HEK293-optimized) *Legend:* **Bold**, STOP codon	ATGGAAAGCGTGCTGGGCTGGGTGTTCCTGGTGGCCATTCTGCAGGGCGTGCAGGGACAGGTGCAGCTGAAGC AGTCTGGCCCTGGACTGGTGCAGCCTAGCCAGAGCCTGAGCATCACCTGTACCGTGTCCGGCTTCAGCCTGAC CAACTACGGCGTGCACTGGGTGCGCCAGAGCCCTGGAAAAGGCCTGGAATGGCTGGGAGTGATTTGGAGCGGC GGCAACACCGACTACAACACCCCCTTCACCAGCAGACTGTCCATCAACAAGGACAACAGCAAGAGCCAGGTGT TCTTCAAGATGAACAGCCTGCAGAGCAACGACACCGCCATCTACTACTGCGCTCGGGCCCTGACCTACTATGA CTACGAGTTCGCCTACTGGGGCCAGGGCACACTCGTGACAGTGTCTGCCGCCACCAGCCAGGACCTGAGCGTG TTCCCTCTGGCCAGCTGCTGCAAGGACAATATCGCCAGCACCAGCGTGACCCTGGGCTGCCTCGTGACCGGCT ACCTGCCTATGAGCACCACAGTGACCTGGGACACCGGCAGCCTGAACAAGAACGTGACCACCTTCCCCACCAC CTTTCACGAGACATACGGCCTGCACAGCATCGTGTCTCAAGTGACCGCCAGCGGCAAGTGGGCCAAGCAGAGA TTCACATGCAGCGTGGCCCACGCCGAGAGCACCGCCATTAACAAGACCTTCAGCGCCTGCGCCCTGAACTTCA TCCCTCCCACCGTGAAGCTGTTCCACAGCAGCTGCAACCCCGTGGGCGATACCCACACCACCATTCAGCTGCT GTGCCTGATCAGCGGCTACGTGCCCGGCGACATGGAAGTGATCTGGCTGGTGGATGGCCAGAAGGCTACCAAC ATCTTCCCCTACACCGCCCCTGGCACCAAAGAGGGCAATGTGACCAGCACCCACTCCGAGCTGAACATCACCC AGGGCGAGTGGGTGTCACAGAAAACCTACACCTGTCAAGTGACATACCAGGGCTTCACCTTCAAGGACGAGGC CCGGAAGTGCAGCGAGAGCGATCCTAGAGGCGTGACCTCCTACCTGAGCCCCCCTAGCCCTCTGGACCTGTAC GTGCACAAGGCCCCCAAGATCACCTGTCTGGTGGTGGACCTGGCCACAATGGAAGGCATGAACCTGACCTGGT ACAGGGAAAGCAAAGAACCCGTGAACCCAGGCCCTCTGAACAAAAAGGACCACTTCAACGGCACCATCACCGT GACAAGCACCCTGCCCGTGAACACCAACGACTGGATCGAGGGCGAGACTTACTACTGCAGAGTGACCCACCCT CATCTGCCCAAGGACATCGTGCGGTCTATCGCCAAGGCCCCAGGCAAAAGGGCCCCTCCCGATGTGTACCTGT TCCTGCCTCCCGAGGAAGAACAGGGCACCAAGGACAGAGTGACACTGACCTGCCTGATCCAGAACTTCTTCCC CGCCGACATCTCCGTGCAGTGGCTGAGAAACGACAGCCCCATCCAGACCGACCAGTACACCACCACCGGCCCT CACAAGGTGTCCGGAAGCAGACCCGCCTTCTTCATCTTCAGCAGGCTGGAAGTGTCCCGGGTGGACTGGGAGC AGAAGAACAAGTTCACATGCCAGGTGGTGCACGAGGCCCTGAGCGGCTCTCGGATCCTGCAGAAATGGGTGTC CAAGACCCCCGGCAAG**TGA**

(Continued)

TABLE E5. (*Continued*)

Sequence description	Sequence
Can225IgE-λ LAMBDA chain amino acid sequence *Legend:* **Bold**, dog immunoglobulin lambda light-chain variable region leader (XP_013963655.1) Underlined, 225 light-chain variable region Regular, dog immunoglobulin lambda constant region	**MTSTMAWSSFLLTLLAHFTGSWA**DILLTQSPVILSVSPGERVSFSCRASQSIGTNIHWYQQRTNGSPRLLIKYASESI SGIPSRFSGSGSGTDFTLSINSVESEDIADYYCQQNNNWPTTFGAGTKLELKRTVAQPKASPSVTLFPPSSEELGANK ATLVCLISDFYPSGVTVAWKADGSPVTQGVETTKPSKQSNNKYAASSYLSLTPDKWKSHSSFSCLVTHEGSTVEKK VAPAECS
Can225IgE-λ LAMBDA light-chain DNA sequence (HEK293-optimized) *Legend:* **Bold**, STOP codon	ATGACCAGCACAATGGCCTGGTCCAGCTTCCTGCTGACCCTGCTGGCCCACTTTACAGGCAGCTGGGCCGACATT CTGCTGACACAGAGCCCCGTGATCCTGAGCGTGTCCCCTGGCGAGAGAGTGTCCTTCAGCTGCAGAGCCAGCCA GAGCATCGGCACCAACATCCACTGGTATCAGCAGCGGACCAACGGCAGCCCCAGACTGCTGATTAAGTACGCCAG CGAGTCCATCAGCGGCATCCCCAGCAGATTTTCCGGCAGCGGCTCCGGCACCGACTTCACCCTGAGCATCAACAG CGTGGAAAGCGAGGATATCGCCGACTACTACTGCCAGCAGAACAACAACTGGCCCACCACCTTCGGAGCCGGCAC CAAGCTGGAACTGAAGAGAACCGTGGCCCAGCCCAAGGCCAGCCCTAGCGTGACACTGTTCCCTCCAAGCAGCGA GGAACTGGGCGCCAACAAGGCCACACTCGTGTGCCTGATCAGCGACTTCTACCCCAGCGGCGTGACCGTGGCCTG GAAGGCTGATGGCTCTCCTGTGACCCAGGGCGTGGAAACCACCAAGCCCAGCAAGCAGTCCAACAACAAATACG CCGCCAGCAGCTACCTGAGCCTGACCCCCGATAAGTGGAAGTCCCACAGCAGCTTCAGCTGTCTCGTGACCCACG AGGGCAGCACCGTGGAAAAGAAAGTGGCCCCTGCCGAGTGCAGC**TGATAG**

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TABLE E6. PIPE primers used for ligation-free cloning can225IgE-λ heavy- and light- chain sequences into pVitro-1 hygro

Primer name	Sequence
can225IgE-λ epsilon forward	TACAGCTAGCTGGCCAGACATGATAAGATACATTGATGAG
can225IgE-λ epsilon reverse	TCCGGATTGCTTTGAATTAGCGGTGGCTTTCACAACACCT
can225IgE-λ lambda forward	AGGGATCCCGTACGCCTAGGAGCAGGTTTCCCCAATGACA
can225IgE-λ lambda reverse	ACCGGTTGCTTTGAATTAGCGGTGGTTTTCACAACACCTA

PIPE, Polymerase incomplete primer extension.